HEPATOLOGY

HEPATOLOGY, VOL. 67, NO. 4, 2018

Therapeutic Inhibition of Inflammatory Monocyte Recruitment Reduces Steatohepatitis and Liver Fibrosis

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Macrophages are key regulators of liver fibrosis progression and regression in nonalcoholic steatohepatitis (NASH). Liver macrophages comprise resident phagocytes, Kupffer cells, and monocyte-derived cells, which are recruited through the chemokine receptor C-C motif chemokine receptor 2 (CCR2). We aimed at elucidating the therapeutic effects of inhibiting monocyte infiltration in NASH models by using cenicriviroc (CVC), an oral dual chemokine receptor CCR2/CCR5 antagonist that is under clinical evaluation. Human liver tissues from NASH patients were analyzed for $CCR2^+$ macrophages, and administration of CVC was tested in mouse models of steatohepatitis, liver fibrosis progression, and fibrosis regression. In human livers from 17 patients and 4 controls, $CCR2^+$ macrophages increased parallel to NASH severity and fibrosis stage, with a concomitant inflammatory polarization of these cluster of differentiation 68^+, portal monocyte-derived macrophages (MoMF). Similar to human disease, we observed a massive increase of hepatic MoMF in experimental models of steatohepatitis and liver fibrosis. Therapeutic treatment with CVC significantly reduced the recruitment of hepatic Ly-$6C^+$ MoMF in all models. In experimental steatohepatitis with obesity, therapeutic CVC application significantly improved insulin resistance and hepatic triglyceride levels. In fibrotic steatohepatitis, CVC treatment ameliorated histological NASH activity and hepatic fibrosis. CVC inhibited the infiltration of Ly-$6C^+$ monocytes, without direct effects on macrophage polarization, hepatocyte fatty acid metabolism, or stellate cell activation. Importantly, CVC did not delay fibrosis resolution after injury cessation. RNA sequencing analysis revealed that MoMF, but not Kupffer cells, specifically up-regulate multiple growth factors and cytokines associated with fibrosis progression, while Kupffer cells activated pathways related to inflammation initiation and lipid metabolism. *Conclusion*: Pharmacological inhibition of $CCR2^+$ monocyte recruitment efficiently ameliorates insulin resistance, hepatic inflammation, and fibrosis, corroborating the therapeutic potential of CVC in patients with NASH. (HEPATOLOGY 2018;67:1270-1283)

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Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in industrialized countries, and especially its progressive inflammatory form, nonalcoholic steatohepatitis (NASH), predisposes to cirrhosis and hepatocellular carcinoma.[1] Due to the rapidly increasing rates of obesity and type 2 diabetes worldwide,[2] NASH is projected to become an enormous clinical and economic burden.[3] In patients with NASH, hepatic fibrosis is

Abbreviations: ALT, alanine aminotransferase; CCL2, chemokine (C-C motif) ligand 2; CCR2, C-C motif chemokine receptor 2; CD, cluster of differentiation; CVC, cenicriviroc; HSC, hepatic stellate cell; MCD, methionine/choline-deficient; MoMF, monocyte-derived macrophage; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

Received May 19, 2017; accepted September 19, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29544/suppinfo.

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Access to the RNA sequencing data is available through the Gene Expression Omnibus, accession no. GSE98782.

Supported by the German Research Foundation (Ta434/5-1and SFB/TRR57), the Interdisciplinary Center for Clinical Research Aachen, and the B. Braun Foundation. Aspects of this work have been supported by the facilities of the Newcastle NIHR Biomedical Research Centre. O.G. and Q.M.A. are members of the EPoS (Elucidating Pathways of Steatohepatitis) consortium funded by the Horizon 2020 Framework Program of the European Union under grant agreement 634413. The study sponsor had no role in the study design or in the collection, analysis, and interpretation of data.

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DOI 10.1002/hep.29544

Potential conflict of interest: Dr. Anstee received grants from AbbVie. Dr. Lefebvre is employed by Allergan. Dr. Tacke consults for and received grants from Tobira and Allergan.

the major characteristic predicting liver-related and overall mortality,[4] leading to the hope that targeting central pathways driving fibrosis progression might provide therapeutic opportunities for the therapy of NAFLD/NASH.[5] One of these core mechanisms that has been identified in patients and mouse models of liver fibrosis is the accumulation of fibrogenic macrophages in the liver.[6] Importantly, hepatic macrophages are heterogeneous cell populations, consisting of liver-resident phagocytes, termed *Kupffer cells*, and monocyte-derived macrophages (MoMF) that are recruited from the circulation to the liver.[7] During acute or chronic injury to the liver, monocytes are massively attracted to sites of hepatic injury, and MoMF represent the dominant macrophage population.[8] Circulating inflammatory monocytes are attracted to the injured liver through their chemokine receptor C-C motif chemokine receptor 2 (CCR2),[9-11] while the corresponding chemokine (C-C motif) ligand 2 (CCL2), or monocyte chemoattractant protein-1, is strongly expressed by various liver cells like activated Kupffer cells or damaged hepatocytes.[12] Infiltrating monocytes differentiate into liver macrophages that promote the activation of hepatic stellate cells (HSCs) to become myofibroblasts, the main source of extracellular matrix, especially collagen, in a chronically inflamed liver.[13]

Although many of the mechanistic concepts are derived from animal models of NASH and liver fibrosis, several observations demonstrate that the same pathways are active in human disease, suggesting their suitability as therapeutic targets. For instance, CCR2/CCL2 is upregulated in fibrotic livers from patients, alongside an accumulation of inflammation-polarized monocyte-derived phagocytes that activate HSCs.[14] Patients with NAFLD have increased levels of the macrophage activation marker soluble cluster of differentiation (CD) 163,[15] underlining the importance of macrophages in the context of chronic liver injury. Moreover, increased proportions of $CCR2^+$ macrophages in visceral adipose tissue were associated with histological disease severity of NASH in obese patients.[16]

Cenicriviroc (CVC) is an orally available, dual inhibitor of the chemokine receptors CCR2 and CCR5, which efficiently inhibits monocyte infiltration.[11,17] CVC is currently being evaluated in a phase 2b clinical trial in patients with NASH and fibrosis,[18] and the first data indicated that patients treated with CVC were twice as likely to show improvement of fibrosis (without worsening of NASH) after only 1 year of a well-tolerated oral therapy.[19] Early *in vivo* studies testing CVC in thioacetamide-induced liver damage in rats and a combined streptozotocin–dietary model in mice (Stelic STAM model) had indicated antifibrotic effects of this drug,[17] but detailed mechanistic data are currently lacking. We hypothesized that CVC's main mechanism of action would be the inhibition of monocyte recruitment, thereby modulating the hepatic macrophage pool toward less inflammatory and less fibrogenic macrophages. In this study, we demonstrate the hepatic accumulation of inflammatory $CCR2^+$ macrophages in patients with NASH fibrosis and show that the dual CCR2/CCR5 inhibitor CVC effectively ameliorates steatohepatitis and fibrosis in mouse models by inhibiting the infiltration of monocytes during chronic liver injury. Thus, our study provides functional evidence for the successful therapeutic targeting of monocyte recruitment in steatohepatitis and fibrosis, mandating the clinical development of CCR2/CCR5 inhibitors in patients with NASH.

Material and Methods

HUMAN PATIENTS AND IMMUNOHISTOCHEMISTRY

This study included patients with biopsy-proven NAFLD diagnosed in the Freeman Hospital,

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TABLE 1. Characteristics of Human NAFLD Patients

	Steatosis	NASH	NASH Fibrosis	NASH Cirrhosis
n	4	5	4	4
Female, n (%)	1 (25%)	4 (80%)	1 (25%)	3 (75%)
Age, median (± IQR), years	51 (± 16.5)	52 (± 2)	58.5 (± 17)	61.5 (± 7.5)
ALT, median (± IQR), IU/L	59.5 (± 29.5)	104 (± 65)	76.5 (± 5)	85.5 (± 22)
AST, median (± IQR), IU/L	37.5 (± 21.5)	53 (± 25)	52.5 (± 14.25)	56 (± 9.25)
BMI, median (± IQR), kg/m^2	32.4 (± 4.7)	38.1 (± 10.9)	37.9 (± 8.2)	36.2 (± 6.1)
Type 2 diabetes, n (%)	1 (25%)	3 (60%)	2 (50%)	2 (50%)
Liver histology*				
Kleiner score steatosis	1.5 (± 1.25)	2 (± 0)	2.5 (± 1.25)	1.5 (± 1)
Kleiner score ballooning	0 (± 0)	1 (± 0)	1 (± 0)	1.5 (± 1)
Kleiner score inflammation	0 (± 0)	2 (± 1)	1 (± 0)	1.5 (± 1)
Kleiner NAS score	1.5 (± 1.25)	5 (± 1)	5 (± 0.75)	4.5 (± 1.25)
Kleiner fibrosis stage	0 (± 0) (4 × F0)	1 (± 0) (5 × F1)	2 (± 0.5) (3 × F2, 1 × F3)	4 (± 0) (4 × F4)

*Data on histological scoring are presented as median ± IQR.
Abbreviations: AST, aspartate aminotransferase; BMI, body mass index; IQR, interquartile range; NAS, NAFLD activity score.

Newcastle upon Tyne, UK, between 1999 and 2015. Seventeen patient samples were selected for this study based on the grade and stage of disease (detailed patient data are shown in Table 1). As a healthy liver control group, distant liver tissue from colorectal metastasis was used (n = 4). Histological semiquantitative steatosis, activity, and fibrosis score were used to determine the degree of steatosis (S0-S3), activity of steatohepatitis (A0-A4), and stage of fibrosis (F0-F4).[20] This study was conducted under the ethical approval of the Newcastle HPB Biobank (10/H0906/41) with all patients having given informed consent.

Formalin-fixed and paraffin-embedded tissue slices, 4 μm thick, were deparaffinized and rehydrated. Primary antibodies were directed against CCR2 (1/100, ab176390; Abcam, Cambridge, UK), CD163 (1/100, CD163-L-CE; Leica Biosystems), S100A9 (1/1000, ab63818; Abcam), or CD68 (ready-to-use, clone KP1; Dako). Immunopositive cells were counted in parenchyma and the portal tract, and the number of positive cells in the portal tract was normalized to the size of the area. A detailed description can be found in the Supporting Information.

ANIMAL EXPERIMENTS

C57BL6/J wild-type mice were purchased from Janvier Labs (France) at the age of 8 weeks and then housed in a specific pathogen–free environment at the Animal Facility of the University Hospital Aachen. All *in vivo* experiments were performed with male mice at 9-18 weeks of age under conditions approved by the appropriate institutional and governmental authorities according to German legal requirements.

INDUCTION OF CHRONIC LIVER INJURY AND PHARMACOLOGICAL TREATMENT

Chronic liver injury was induced by feeding a Western diet (Ssniff, Soest, Germany; product no. E15723-34) for 16 weeks and a methionine/choline-deficient (MCD) diet (Ssniff; product no. E15653-94) for 8 weeks. Regression from fibrosis was studied by switching from the MCD diet to a chow diet after 8 weeks.[21] CVC was dispensed in sterile water mixed with 0.5% methylcellulose (400 cps) and 1% Tween-80.[11] Western diet–fed mice received CVC in the drinking water at a concentration of 0.42 mg/mL from the beginning of week 9. In MCD diet-induced liver injury, CVC (100 mg/kg body weight) or an equal amount of vehicle was administered once daily by oral gavage during the last 4 weeks of the 8-week MCD diet–induced injury. In regressive experimental setups, CVC (50 mg/kg body weight) or vehicle was administered for up to 5 days two times a day by oral gavage after 8 weeks of MCD diet–induced injury.

PHENOTYPIC ASSESSMENT

Conventional hematoxylin–eosin or sirius red staining was performed according to established protocols.[22] Sirius red area fractions were analyzed and quantified (ImageJ). Colorimetric tests for hydroxyproline were conducted as described.[10] The NAFLD activity score was assessed by an expert pathologist (T. Longerich) who was blinded to the treatment group. Immunohistochemistry for F4/80 (Abcam) was performed on paraffin-

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embedded liver sections and indirect immunofluorescence staining with collagen-1 antibody (Novus Biologicals, Germany) on liver cryosections, both according to standard protocols.[23] Hepatic leukocytes were analyzed by multicolor flow cytometry using an LSR-Fortessa (BD Biosciences), as described.[11]

RNA SEQUENCING ANALYSIS OF HEPATIC MACROPHAGES

Hepatic macrophages were isolated from MCD diet–fed mice and sorted by fluorescence-activated cell sorting using an Aria-II (BD Biosciences). Total RNA of MoMF and Kupffer cells was isolated directly afterward. Complementary DNA synthesis and sequencing were performed by Illumina Sequencing (HiSeq, single reads, 1×125 bp; Fasteris SA, Switzerland). Data were analyzed with an in-house pipeline embedded in the workflow management system of the QuickNGS-Environment.[24] Differentially expressed genes (fold change > 2.3, $P < 0.001$) were subsequently used for gene set enrichment analysis (gene ontology biological process, false discovery rate <0.01) using the Cytoscape[25] plug-ins BinGO,[26] Enrichment Map[27] ($P < 0.001$ and false discovery rate Q value <0.001), and Word Cloud.[28]

STATISTICS

All experimental data from mice are presented as mean ± SD. Differences between groups were evaluated by two-tailed unpaired Student *t* test (experimental data) or Mann-Whitney test (human samples) (GraphPad Prism; GraphPad Software).

Additional information is provided in the Supporting Information.

Results

NASH PATIENTS WITH FIBROSIS SHOW SIGNIFICANTLY INCREASED HEPATIC CCR2$^+$ INFLAMMATORY MACROPHAGE

The CCR2-dependent recruitment of inflammatory monocytes has been described in dietary models of experimental steatohepatitis in mice,[9,29,30] and the accumulation of CD68$^+$ macrophages correlates with NASH fibrosis stage in humans.[31] In order to verify the relevance of the CCR2–CCL2 pathway in human NAFLD, we analyzed liver biopsies of 4 controls and 17 patients of different disease severity ranging from steatosis (n = 4) to NASH (n = 5), NASH fibrosis (defined as stages F2 and F3, n = 4), and ultimately NASH cirrhosis (n = 4) (Table 1). CCR2$^+$ macrophages were seen in the portal tract area in all disease stages of NAFLD (Fig. 1A). Kupffer cells showed no positivity for CCR2. Strikingly, the number of CCR2$^+$ monocyte-derived cells in the portal tracts was greatly enlarged in patients with NASH and fibrosis or cirrhosis (Fig. 1A), the NAFLD stages considered at highest medical need for therapeutic interventions.[32] To exclude a bias in quantification due to variation in size of the portal tracts, the absolute number of positive cells was normalized to the area in which they were counted. A significant increase in CCR2$^+$ cells was noted in cirrhosis and fibrosing steatohepatitis compared to control, steatosis, or steatohepatitis (Fig. 1B). This effect was particularly evident in end-stage cirrhosis, where CCR2$^+$ cells were seen to infiltrate the parenchyma. In addition, an increase of S100A9$^+$ macrophages was seen with progression of the disease, suggesting that CCR2$^+$ cells have an inflammatory phenotype.[11] On the other hand, Kupffer cells, as indicated by the marker CD68, did not substantially differ between the stages of NAFLD and were mainly located in the parenchyma as opposed to the portal tracts (Fig. 1A,B). Kupffer cells expressed CD163, a marker indicating anti-inflammatory ("M2-type") properties,[11] which were found to be increased in the portal tract in patients with fibrotic or cirrhotic stage (Supporting Fig. S1A,B). Additional immunofluorescence costaining for CCR2 and CD68 in patients with cirrhotic NASH revealed that CCR2$^+$ infiltrating cells express CD68, thereby underlining the importance of CCR2-dependent macrophage recruitment during NAFLD progression (Supporting Fig. S1C). Collectively, human biopsy analyses revealed the intense infiltration of CCR2$^+$, inflammatory monocyte-derived phagocytes in portal tracts of patients with NASH and fibrosis or cirrhosis, corroborating the concept of pharmacologically targeting this mechanism by CCR2 chemokine receptor antagonism.

CVC INHIBITS HEPATIC MoMF ACCUMULATION AND AMELIORATES STEATOHEPATITIS IN WESTERN DIET–INDUCED LIVER INJURY

To investigate the efficacy of CVC of inhibiting monocyte infiltration in the context of NASH

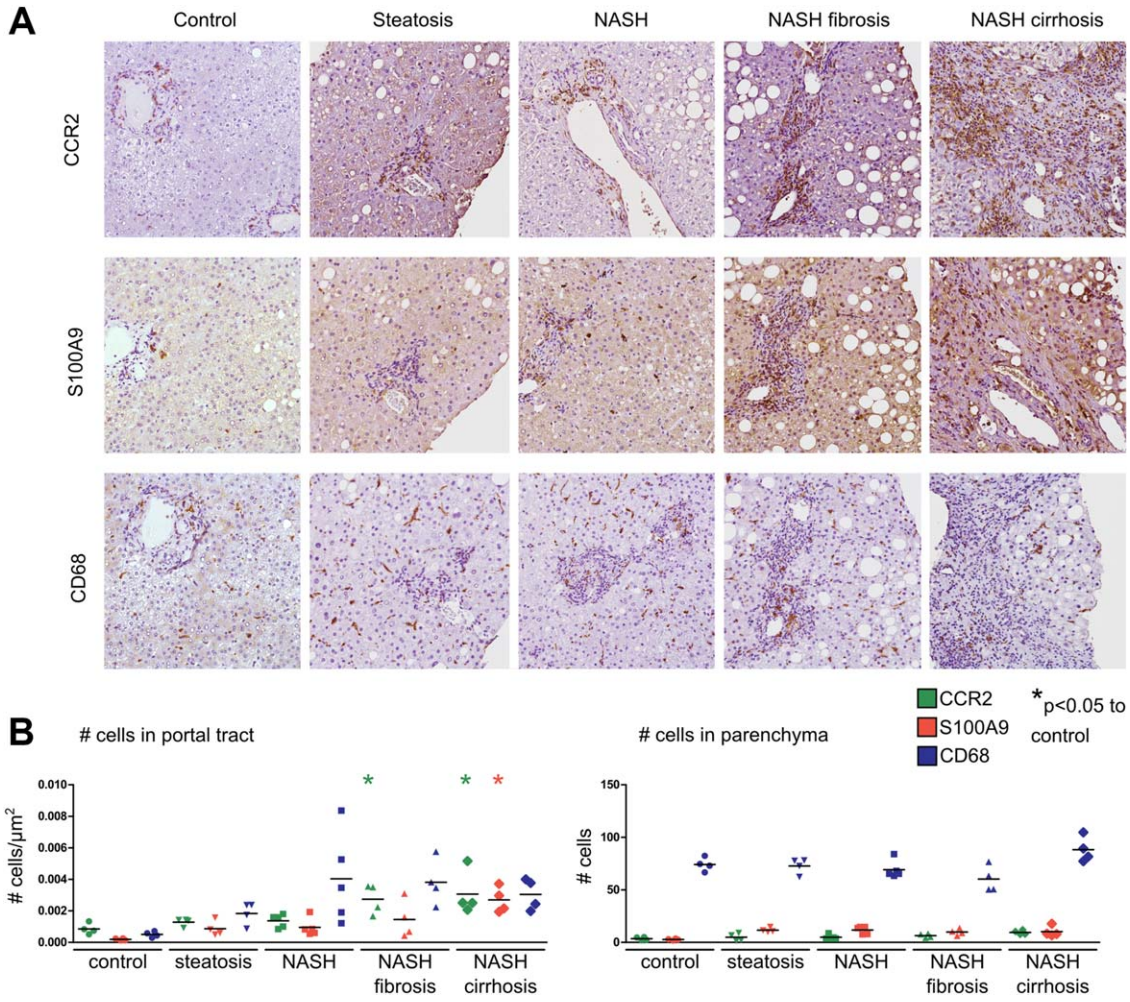

FIG. 1. Livers from NAFLD patients show increased portal CCR2$^+$ inflammatory macrophages. (A) Representative sections of liver samples (n = 21) from patients with different stages of NAFLD (n = 17) and controls (n = 4) that were stained for CCR2, the inflammatory macrophage polarization marker S100A9, and the general macrophage marker CD68. Magnification × 200. (B) Quantification of immunohistochemical staining for the portal tract (number of cells per portal tract area, to exclude interpatient differences) as well as for the liver parenchyma. For each individual one high-power field was counted for the portal tract and three for the parenchyma. Data from individual patients are presented. *$P < 0.05$ compared to the control group (Mann-Whitney U test).

progression, we chose a murine model with a high-fat, high-sugar, and high-cholesterol Western diet. Western diet feeding is a commonly used physiologic dietary model in which mice show typical changes reflecting human metabolic syndrome, such as obesity, lipometabolic disorder, impaired glucose tolerance, and insulin resistance, in conjunction with NAFLD.[33] Mice were fed a Western diet over a period of 16 weeks and received CVC or vehicle control solution in the drinking water from the beginning of week 9 (Fig. 2A). Efficient dosing of CVC was confirmed by measuring serum CCL2 levels, which are elevated in response to CCR2 inhibition by CVC (Fig. 2A). CVC treatment significantly reduced steatohepatitis and fatty degeneration of the liver as seen by reduced alanine aminotransferase (ALT) levels and histology (Fig. 2A,B). Most strikingly, CVC treatment reduced the number of hepatic macrophages to approximately the number in chow diet control animals, as assessed by F4/80 immunohistochemistry and flow-cytometric analysis (Fig. 2C,D; Supporting Fig. S2A). Flow-cytometric analysis revealed that only MoMF, but not Kupffer cells, were reduced upon CVC treatment (Fig. 2D,E). The reduction of hepatic MoMF was accompanied by

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a significant reduction in the gene expression of *Ccr2* and *Ccr5* in total liver (Fig. 2D; Supporting Fig. S2B), whereas the expression levels of the corresponding chemokines *Ccl2* and *Ccl5* were unaltered (Supporting Fig. S2B). In conjunction with reduced MoMF, CVC-treated animals showed a reduction of hepatic triglyceride levels (Fig. 2E) and an improvement in glucose tolerance (Fig. 2F). Food intake, body weight, and adipose tissue inflammation remained unaltered by CVC (Supporting Fig. S2C; data not shown), while hepatic fibrosis was not observed in the 16-week Western diet model (Supporting Fig. S2D). Although CVC inhibits not only CCR2 but also CCR5, the composition of intrahepatic lymphocytes remained unaffected in livers of chow diet–treated or Western diet–treated animals (Supporting Fig. S2E). Reduction of Ly-6C$^+$ MoMF by CVC was accompanied by increased expression of anti-inflammatory (CD206 and CD301) and immunoregulatory (CD274) markers on liver MoMF (Supporting Fig. S2F). These data indicate that inhibition of infiltrating MoMF reduces hepatic inflammation, which improves hallmark characteristics of NASH progression such as triglyceride deposition and insulin resistance.

INHIBITING MONOCYTE INFILTRATION AMELIORATES FIBROSIS AND STEATOHEPATITIS EXPERIMENTAL LIVER INJURY

In mice and humans, infiltration of inflammatory monocytes into the chronically injured liver is closely associated with progression of hepatic fibrosis.[10] In order to investigate the effects of CVC on fibrosis progression, we employed the MCD diet liver injury model, which is characterized by severe hepatic inflammation and fibrosis.[34] Mice were fed the MCD diet over a period of 8 weeks, and CVC or vehicle control solution was administered once per day by oral gavage from half of the time of injury progression (Fig. 3A). CVC treatment strongly and specifically inhibited the monocyte infiltration in MCD diet–challenged mice (Fig. 3B; Supporting Fig. S3A,B), which was accompanied by significantly reduced liver fibrosis (Fig. 3C). These effects were accompanied by significantly reduced hepatocyte ballooning, resulting in a 1-point reduction of the NAFLD activity score (Supporting Fig. S3A,B). Gene expression of both *Ccr2* and *Ccr5* was significantly reduced in CVC-treated mice (Fig. 3B), whereas expression of the ligands Ccl2 and Ccl5 remained unaltered by CVC (Supporting Fig. S3C). Efficient CCR2 inhibition was again confirmed by increased levels of serum CCL2 in the CVC-treated group (Supporting Fig. S3D).

While these data from two experimental steatohepatitis models indicated that CVC mainly inhibited monocyte recruitment to the liver, we also aimed at excluding alternative mechanisms of action. We tested the effects of CVC on primary murine hepatocytes and HSCs *in vitro*. CVC did not alter fatty acid uptake of primary mouse hepatocytes, as assessed by exposure to oleic and palmitic acids, followed by subsequent oil red O staining and analysis of lipogenesis pathways (Fig. 3D; additional data not shown). Moreover, CVC did not influence the spontaneous or the transforming growth factor-beta–induced activation of HSCs, as determined by gene expression for alpha-smooth muscle actin and collagen-1 protein secretion (Fig. 3F; additional data not shown). Thus, we conclude that the reduced fibrosis development in experimental steatohepatitis by CVC is the consequence of inhibiting hepatic MoMF recruitment, a main driver of hepatofibrogenesis.[7,10]

WHOLE-GENOME RNA SEQUENCING REVEALS THE PROFIBROTIC POLARIZATION OF MoMF IN NASH

To better understand the role of different hepatic macrophage populations in promoting the progression from NAFLD to NASH and liver fibrosis, we isolated MoMF and Kupffer cells from livers of mice subjected to the MCD diet for 4 weeks and performed mRNA sequencing analysis of these hepatic macrophage populations. Differential gene expression analysis of selected genetic markers showed that both MoMF and Kupffer cells up-regulate the expression of inflammatory chemokines and cytokines as well as extracellular matrix–modulating proteins (Fig. 4A). In particular, MoMF from steatotic livers showed strongly induced expression of genes related to phagocytosis, like *Marco, Mertk*, or *Cd5l*, as well as fibrosis-associated growth factors, like *Vegfa* or *Igf1* (Fig. 4A). Phagocytosis is a mechanism to imprint distinct pathways of tissue macrophages[35] and has been linked to fibrosis resolution in the liver.[36] MoMF and Kupffer cells both up-regulate inflammatory cytokines such as *Il1b* and *Tnfa*, which have been shown to promote the survival of HSCs and thereby to contribute to liver fibrosis.[37] Besides chemokines and cytokines, Kupffer cells also

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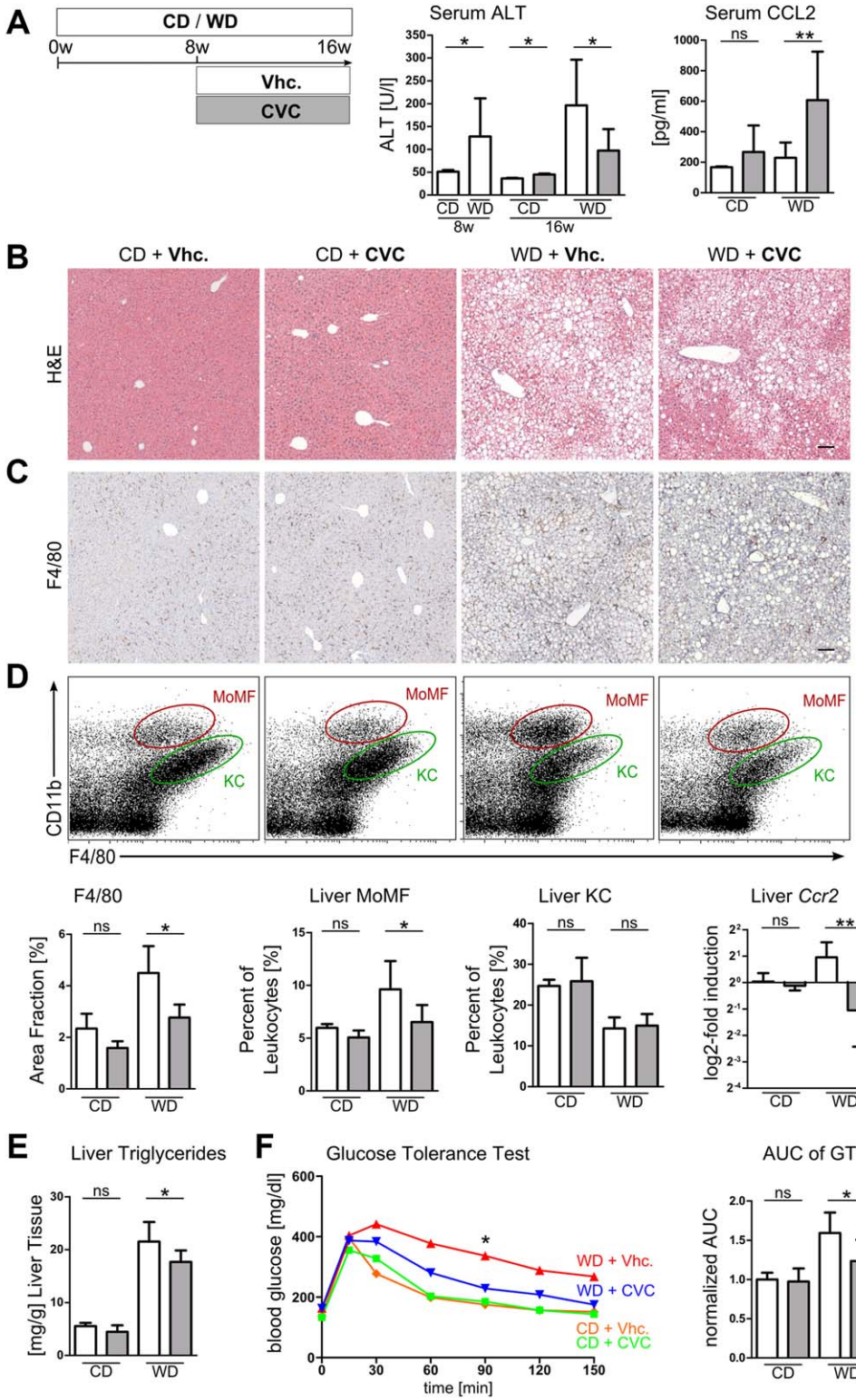

FIG. 2. CVC inhibits hepatic MoMF accumulation and ameliorates steatohepatitis in Western diet–induced liver injury in mice. (A) Steatohepatitis was induced in C57BL/6 wild-type mice by feeding a Western diet over 16 weeks; chow diet–fed mice served as controls. CVC or vehicle treatment was started at week 9 in the drinking water. Serum ALT levels are shown as a marker of hepatocyte necrosis. Serum levels of CCL2 as quantified by enzyme-linked immunosorbent assay. (B) Hematoxylin and eosin staining of representative liver sections. (C) Representative F4/80 immunohistochemical staining of liver sections. (D) Representative flow-cytometric plots showing MoMF (red) and Kupffer cells (green). F4/80-positive area fraction as determined from immunohistochemistry, quantification of MoMF and Kupffer cells in percent of liver leukocytes by fluorescence-activated cell sorting analysis, and gene expression analysis of *Ccr2* from total liver tissue. (E) Liver triglycerides shown as milligrams per gram of liver tissue. (F) Blood glucose levels following intraperitoneal application of 40% sterile glucose solution and normalized area under the curve of the glucose tolerance test. All images were taken at ×10 magnification; scale bar, 100 μm. All data are presented as mean ± SD (n = 4 for untreated, n = 6-8 for experimental groups). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student *t* test). Abbreviations: AUC, area under the curve; CD, chow diet; GTT, glucose tolerance test; H&E, hematoxylin and eosin; KC, Kupffer cell; Vhc, vehicle; WD, Western diet.

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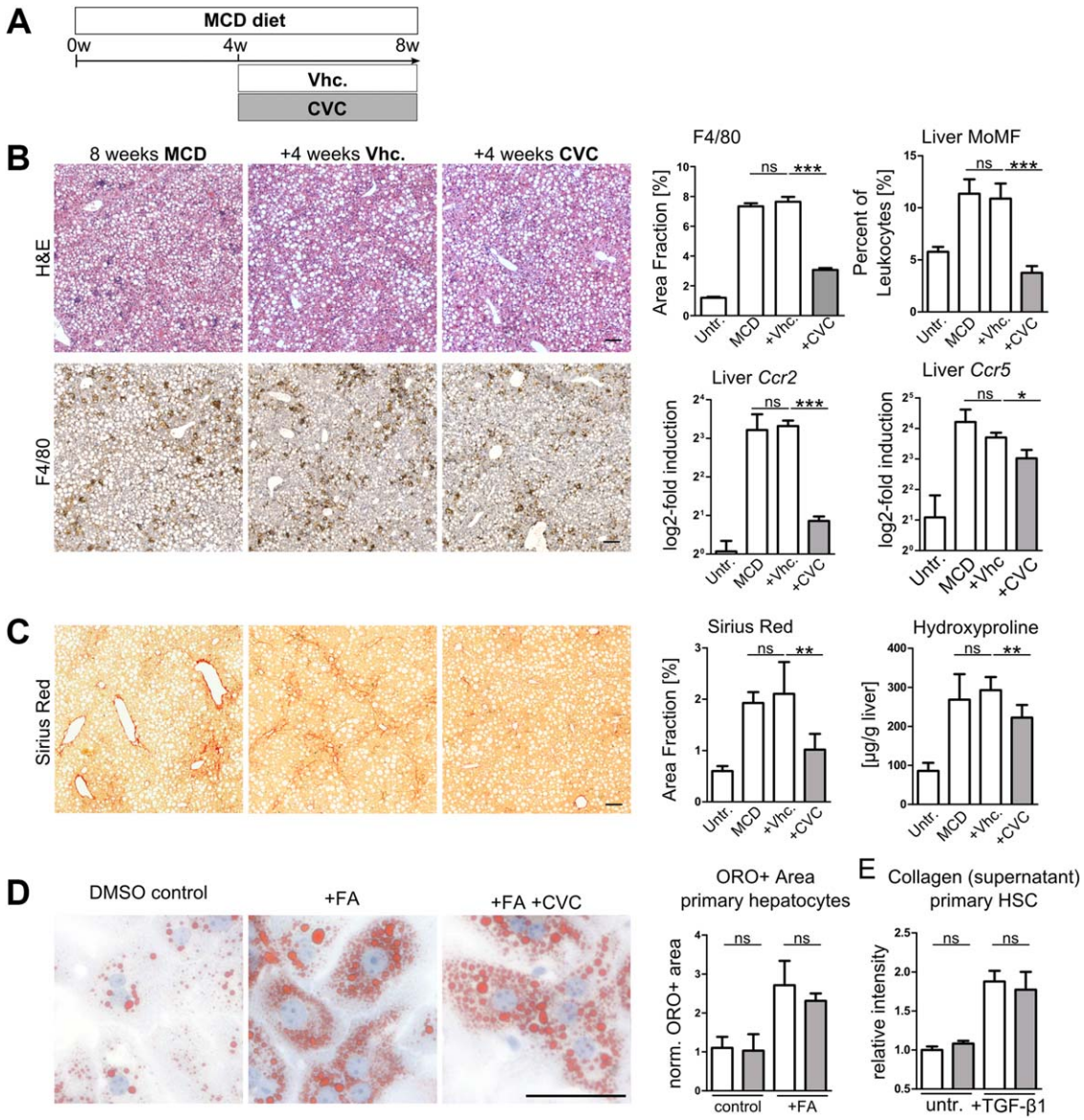

FIG. 3. Therapeutic application of CVC improves fibrosis and ameliorates steatohepatitis in mice. (A) Fibrosis and steatohepatitis were induced in C57BL/6 wild-type mice by feeding an MCD diet over 8 weeks. CVC or vehicle treatment was started once daily orally at half of the time of injury progression. (B) Liver histology (hematoxylin and eosin staining) and F4/80 immunohistochemical staining for macrophages. Liver MoMF were quantified by fluorescence-activated cell sorting analysis. Analysis of the gene expression of *Ccr2* and *Ccr5* in total liver tissue. (C) Liver fibrosis was assessed by quantification of sirius red–positive area fraction and hepatic hydroxyproline content. Images were taken at ×10 magnification; scale bars, 100 µm. (D) Primary mouse hepatocytes were cultured with a mixture of fatty acids for 24 hours, either with or without CVC. Representative images of oil red O–stained primary hepatocytes 24 hours after stimulation, taken at ×20; scale bar, 100 µm. Normalized oil red O–positive area (= fat droplets) of hepatocytes. (E) Collagen-1 protein in the supernatant of activated ultrapure fluorescence-activated cell–sorted primary murine HSCs. HSCs were cultured for 3 days with or without CVC (1 µM), both unstimulated or stimulated with transforming growth factor-β1 (1 ng/mL). All data are presented as mean ± SD (n = 3 for untreated, n = 6-8 for experimental groups for A-C and n ≥ 3 for D and E). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student *t* test). Abbreviations: DMSO, dimethyl sulfoxide; FA, fatty acid; H&E, hematoxylin and eosin; ns, nonsignificant; ORO, oil red O; TGF-β1, transforming growth factor-β1; Untr., untreated; Vhc, vehicle.

expressed genes linked to lipid binding and metabolism such as *Cd36* or *Pparg* (Fig. 4A). Among the most strongly regulated genes between MoMF and Kupffer cells, we found characteristic differentiation markers like *Clec4f* and *Timd4* for Kupffer cells or *Ccr2* for MoMF (Fig. 4B). Interestingly, MoMF highly

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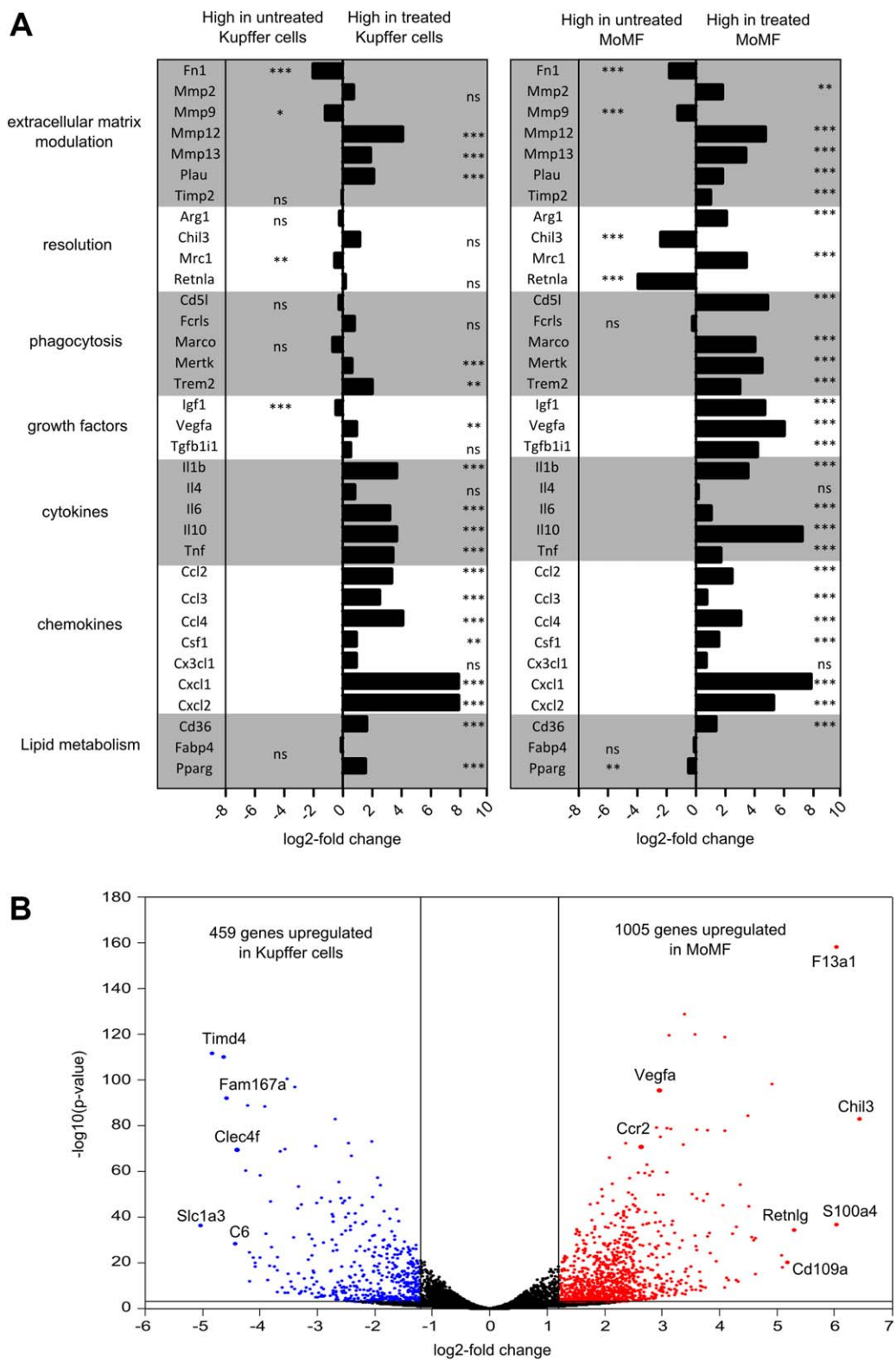

FIG. 4. Whole-genome RNA sequencing reveals the profibrotic polarization of MoMF in experimental NASH. (A) Fluorescence-activated cell–sorted Kupffer cells ($CD11b^{+}$ and $F4/80^{++}$) and MoMF ($CD11b^{++}$ and $F4/80^{+}$) isolated from treated (MCD diet) and untreated (control, chow diet) mice. Purity was > 95%. Differential gene expression of selected marker genes between treated versus untreated Kupffer cells and MoMF is displayed as log2-fold change. (B) Volcano plot showing the differential gene expression between MoMF (red) and Kupffer cells (blue) in MCD diet–fed mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (adjusted P value from DESeq2). Abbreviation: ns, nonsignificant.

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express *Chil3* and *Retnlg*, two markers that are broadly associated with an anti-inflammatory phenotype. Thus, inflammatory cytokines and chemokines are up-regulated in both populations, whereas MoMF highly express various growth factors, equipping them to promote angiogenesis and liver fibrosis.

GENE ONTOLOGY ANALYSIS INDICATES DISTINCT FUNCTIONS OF MoMF AND KUPFFER CELLS IN NASH PROGRESSION

As therapeutic application of CVC mainly affects monocyte infiltration, but not Kupffer cells, we wanted to further dissect the functional differences between MoMF and Kupffer cells. We therefore generated clusters of gene ontology pathways enriched with up-regulated genes ($P < 0.01$) in MoMF compared to Kupffer cells isolated from livers of MCD diet–fed mice (Fig. 5A,B). Modulation of the extracellular matrix and the promotion of angiogenesis were mainly, but not solely, mediated by MoMF (Fig. 5A). In homeostasis, Kupffer cells are known to maintain tolerance toward self-antigens by suppressing T-cell activation, which is disrupted upon liver injury.[38] Interestingly, T-cell activation signals were induced in MoMF, supporting their role for immunogenic activation of T cells during liver injury.[38] In line with previous findings,[7] Kupffer cells expressed various chemokines such as *Ccl2* and *Cxcl1*, which are important mediators of monocyte and neutrophil recruitment.[12] Interestingly, Kupffer cells also up-regulated genes related to lipid metabolism, most likely as a counterbalancing mechanism in response to lipid accumulation in the liver. Moreover, both MoMF and Kupffer cells showed aspects of a complex injury-related phenotype by expressing inflammatory (*Il6, Ptgs2*) as well as anti-inflammatory (*Il10, Chil3*) markers (Fig. 5A,B). The mixed inflammatory phenotype of MoMF and Kupffer cells aligns with findings that macrophage activation patterns are complex and do not fit into a simple inflammatory ("M1") or anti-inflammatory ("M2") macrophage polarization.[39] In fact, CVC primarily affected the number of MoMF in livers of MCD diet–fed or Western diet–fed animals, with only minor effects on the expression of inflammatory (major histocompatibility complex II, CD68), anti-inflammatory (CD206, CD301), or immunoregulatory (CD274) marker expression of hepatic MoMF. Nonetheless, hepatic MoMF expressed lower levels of Ly-6C, consistent with a reduced recruitment of Ly-6C^{high} monocytes (Supporting Figs. S2F and S3F). Taken together, MoMF and Kupffer cells display distinct activation profiles in experimental NASH, supporting that Kupffer cells primarily regulate inflammation and participate in metabolism, while MoMF promote fibrosis and angiogenesis.

INHIBITION OF MONOCYTE INFILTRATION BY CVC DOES NOT AFFECT REPAIR PROCESSES DURING REGRESSION FROM LIVER FIBROSIS

Ly-6C$^+$ MoMF can switch their phenotype toward restorative, Ly-6C low expressing macrophages that are essential for reversal of fibrosis and full injury resolution.[40] Because MoMF, but not Kupffer cells, up-regulated gene markers associated with phagocytosis and liver injury resolution (Fig. 4A), we tested whether inhibition of hepatic monocyte recruitment could impair fibrosis regression. Thus, we fed mice the MCD diet for 8 weeks and afterward started CVC treatment twice daily for 5 days (Fig. 6A). Recovery from MCD diet–induced liver injury and fibrosis was not affected by CVC (Fig. 6B). Importantly, CVC did not affect normalization of ALT levels (Fig. 6B), NAFLD activity score (not shown), or fibrosis regression as assessed by hydroxyproline (not shown) or sirius red staining (Fig. 6C). While CVC treatment moderately affected the recruitment of Ly-6C$^+$ MoMF during recovery in both models, it did not affect the number of Ly-6C$^-$ (restorative) macrophages, nor did it hamper proper fibrosis resolution (data not shown). We therefore conclude that inhibition of monocyte infiltration by CVC does not affect repair processes during regression from liver fibrosis.

Discussion

Chronic inflammation within the liver is tightly linked to fibrosis in virtually all types of liver disease and in experimental models of NASH and liver fibrosis.[6] Based on experimental and clinical data linking the recruitment of monocyte-derived, inflammatory macrophages in the liver to progression of NASH and fibrosis,[12,41] we hypothesized that inhibiting hepatic MoMF accumulation by the chemokine receptor CCR2/CCR5 inhibitor CVC bears therapeutic

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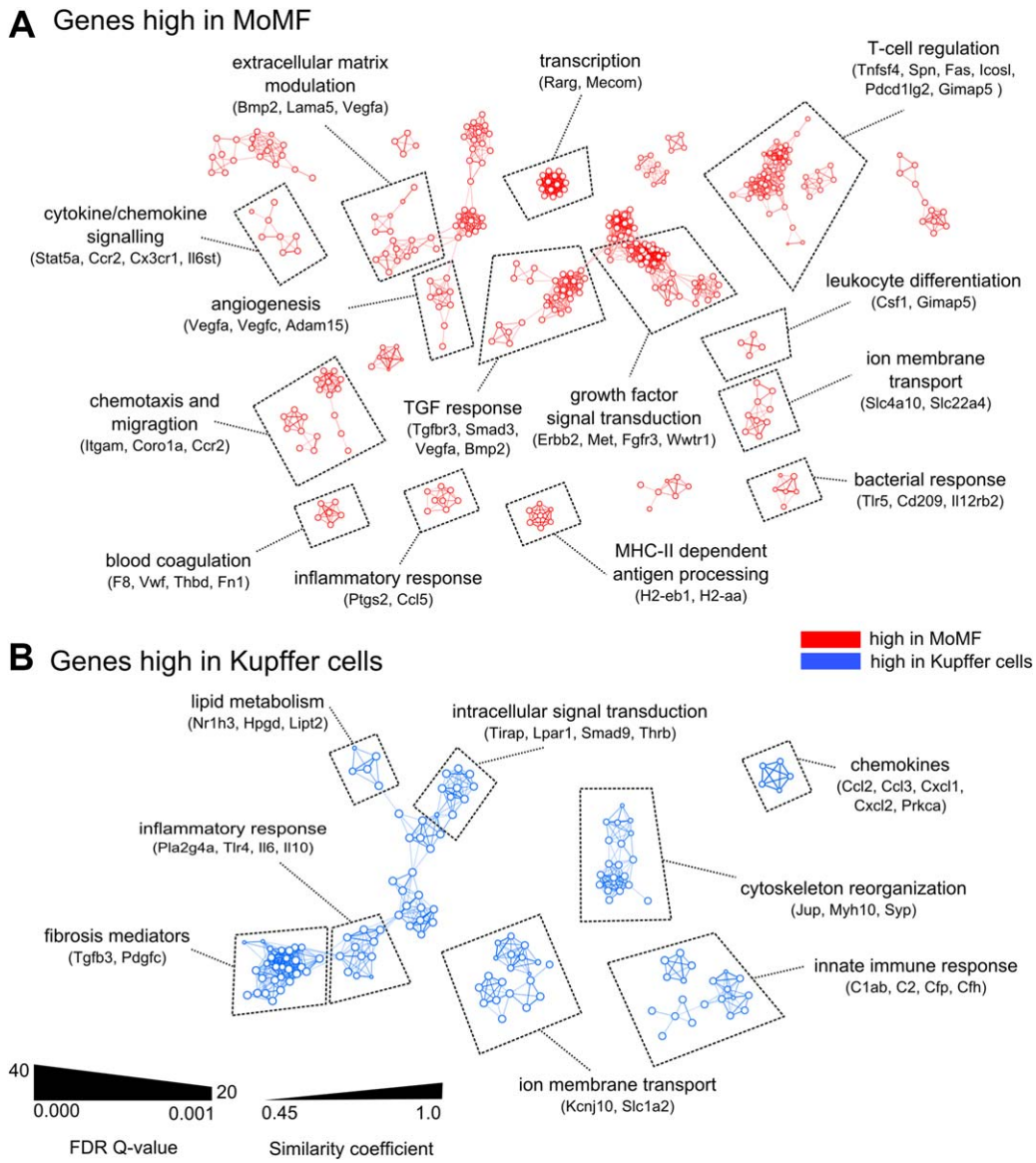
A Genes high in MoMF
extracellular matrix modulation (Bmp2, Lama5, Vegfa)
transcription (Rarg, Mecom)
T-cell regulation (Tnfsf4, Spn, Fas, Icosl, Pdcd1lg2, Gimap5)
cytokine/chemokine signalling (Stat5a, Ccr2, Cx3cr1, Il6st)
angiogenesis (Vegfa, Vegfc, Adam15)
leukocyte differentiation (Csf1, Gimap5)
ion membrane transport (Slc4a10, Slc22a4)
chemotaxis and migragtion (Itgam, Coro1a, Ccr2)
TGF response (Tgfbr3, Smad3, Vegfa, Bmp2)
growth factor signal transduction (Erbb2, Met, Fgfr3, Wwtr1)
bacterial response (Tlr5, Cd209, Il12rb2)
blood coagulation (F8, Vwf, Thbd, Fn1)
inflammatory response (Ptgs2, Ccl5)
MHC-II dependent antigen processing (H2-eb1, H2-aa)
high in MoMF
high in Kupffer cells
B Genes high in Kupffer cells
lipid metabolism (Nr1h3, Hpgd, Lipt2)
intracellular signal transduction (Tirap, Lpar1, Smad9, Thrb)
chemokines (Ccl2, Ccl3, Cxcl1, Cxcl2, Prkca)
inflammatory response (Pla2g4a, Tlr4, Il6, Il10)
cytoskeleton reorganization (Jup, Myh10, Syp)
fibrosis mediators (Tgfb3, Pdgfc)
innate immune response (C1ab, C2, Cfp, Cfh)
ion membrane transport (Kcnj10, Slc1a2)
40
20
0.000
0.001
FDR Q-value
0.45
1.0
Similarity coefficient

FIG. 5. Gene ontology analysis displays different roles for MoMF and Kupffer cells in experimental NASH. (A) Clustered gene ontology pathways enriched with up-regulated genes ($P < 0.01$) in MoMF compared to Kupffer cells isolated from livers of MCD diet–fed mice. (B) Enrichment map of significantly up-regulated gene ontology pathways in Kupffer cells isolated from MCD mice. n = 2 per population and condition. Abbreviations: FDR, false discovery rate; MHC, major histocompatibility complex; TGF, transforming growth factor.

potential. In our study, we confirmed the clinical relevance of chemokine-dependent macrophage accumulation in a cohort of patients with NASH, demonstrating an association between CCR2-expressing, inflammation-polarized portal macrophages with the fibrosis and cirrhosis stages of NASH. Consequently, inhibition of monocyte infiltration by CVC reduced steatohepatitis and fibrosis in experimental mouse models of chronic liver injury.

In order to address our research question, we employed two models of NASH and inflammation, the Western and MCD diets. Western diet feeding induces a phenotype of obesity, steatosis, and (some degree of) steatohepatitis, which closely resembles human pathology of fatty liver disease but lacks severe fibrosis. The model of MCD diet–induced liver injury lacks certain hallmark characteristics of human NAFLD pathology (like obesity or insulin resistance);

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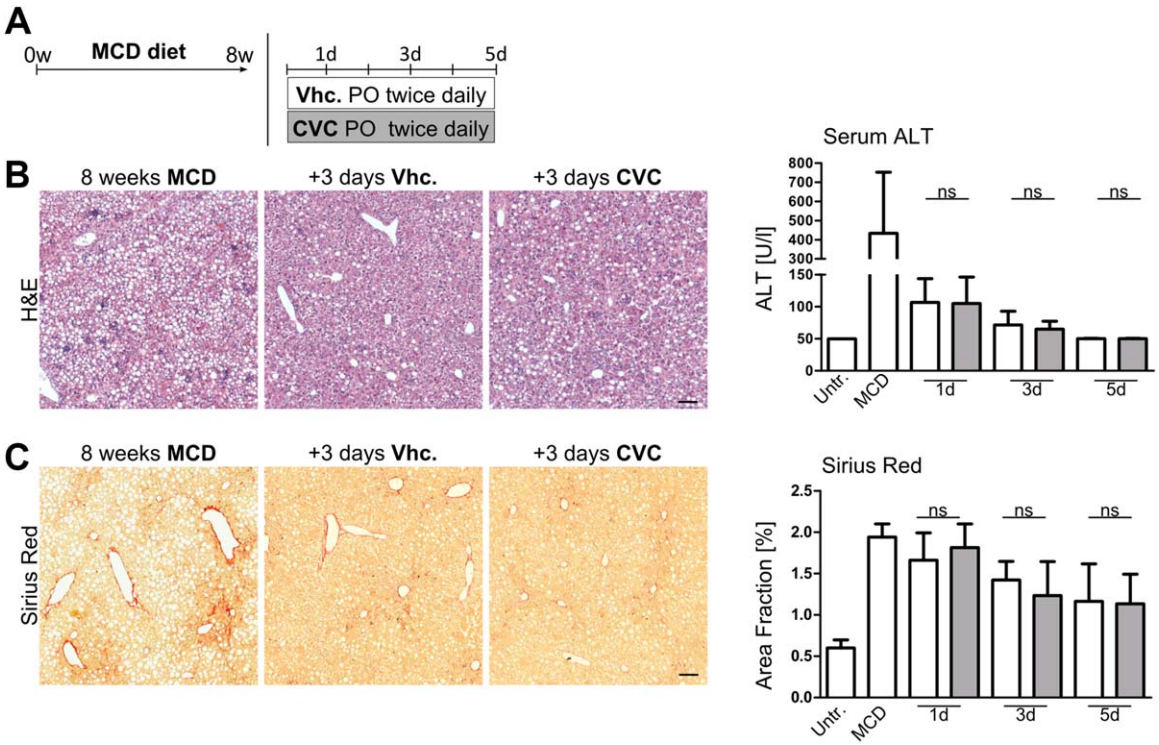

FIG. 6. Inhibition of monocyte infiltration does not affect repair processes during regression from experimental liver fibrosis. (A) Steatohepatitis and fibrosis were induced by feeding an MCD diet to C57BL/6 wild-type mice for 8 weeks. Regression was induced by switching to a chow diet, alongside treatment with CVC. (B) Liver histology (hematoxylin and eosin staining) and serum ALT levels. (C) Sirius red staining and quantification of sirius red–positive area fraction. All images were taken at ×10 magnification; scale bar, 100 μm. All data are presented as mean ± SD (n = 3 for untreated, n = 6-8 for experimental groups). $^*P < 0.05$ (unpaired Student *t* test). Abbreviations: H&E, hematoxylin and eosin; ns, nonsignificant; PO, orally (*per os*); Untr., untreated; Vhc, vehicle.

however, it induces severe liver inflammation and fibrosis. To comprehensively assess the effects of CVC on both NAFLD and liver fibrosis, we therefore employed both models side by side in our study. We started pharmacological treatment after half the time of total injury induction in order to evaluate the therapeutic efficacy during fibrosis progression. Importantly, the plasma half-life of CVC is much shorter in mice (~2 hours) than in humans (30-40 hours),[17] indicating that a once-daily dosing by gavage in mice was likely suboptimal in both models.

We previously reported that monocyte infiltration into the liver can be effectively inhibited by blocking the CCR2 ligand CCL2, using the RNA aptamer molecule mNOX-E36.[9,11] Experimental data also suggest that CCR2 inhibition is useful for treating NASH and liver fibrosis.[29,42] While it remains open whether CCR2 or CCL2 represents the better target, CVC has the advantages of oral administration and a very favorable safety profile.[17] We confirmed effective dosing by measuring CCL2 serum levels, which are significantly increased upon pharmacological blockade of CCR2. However, CVC blocks not only CCR2 but also CCR5, which has been implicated in the migration, activation, and proliferation of matrix-producing HSCs.[43,44] CCR5 is also important for the recruitment of lymphocyte populations (natural killer cells, T cells) to the liver, but this has been primarily related to conditions of viral or (auto-)immune hepatitis.[12] Most interestingly, we found that hepatic gene expression of both *Ccr2* and *Ccr5* was significantly reduced upon CVC treatment, although the strongest reduction could be observed for *Ccr2*. The reduction of *Ccr2* expression most likely reflected the reduced influx of $CCR2^+$ monocytes. In contrast, CVC did not interfere with (CCR5-expressing) lymphocyte populations, in line with prior observations in models of acute liver injury.[11] However, it remains unclear whether a higher dose of CVC (e.g., by intravenous or intraperitoneal application) might confer stronger inhibitory effects on CCR5-expressing cells, possibly even including hepatocytes or HSCs, as well. In the

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conditions of our study, CVC's antifibrotic effects in mice were principally driven by inhibiting the intrahepatic accumulation of MoMF through CCR2.

The view on macrophages in the liver has drastically changed in recent years as it became apparent that hepatic macrophages consist of heterogeneous populations in liver health and disease.[8] Principally, a self-sustaining, locally proliferating, and mainly tolerogenic population of Kupffer cells[38,45,46] can be distinguished from monocyte-derived, infiltrating macrophages ("MoMF"), which are rather immunogenic and receive signals prompting their functional differentiation from the local microenvironment.[23,46-48] In our study, inhibition of infiltrating macrophages by CVC was associated with reduced fibrosis and hepatocyte ballooning. It has been described that monocyte-derived cells can activate HSCs in the context of chronic liver injury in mice and humans by secreting fibrotic factors such as transforming growth factor-beta or platelet-derived growth factor[10,14] as well as by prolonging HSC survival through interleukin-1 and tumor necrosis factor.[37] We found that during NASH MoMF highly express genes that are associated with modulation of the extracellular matrix as well as growth factors that can promote both fibrosis and angiogenesis. The up-regulation of markers linked to phagocytosis indicates the importance of MoMF in contributing to clearing cell debris, which is a hallmark feature in the resolution of liver fibrosis.[36] After discontinuation of injury, the recruitment of bone marrow–derived monocytes into the liver rapidly declines,[21,36] and the existing macrophages in the liver undergo a functional shift toward restorative macrophages.[40] In our study, pharmacological treatment with CVC during fibrosis regression affected neither the number of hepatic restorative macrophages nor the resolution of liver fibrosis in mice.

CVC (at a dose of 150 mg once daily) is currently being tested in a phase 2b clinical trial in adult patients with NASH and fibrosis (CENTAUR study 652-2-203; NCT02217475).[17] No drugs are currently approved for the treatment of NASH,[49] but many agents are under investigation.[5] These agents, like the farnesoid X receptor agonist obeticholic acid or the peroxisome proliferator–activated receptor α/δ agonist elafibranor, have different molecular and cellular targets.[13] The unique mode of CVC's antifibrotic action, i.e., inhibiting the recruitment of monocyte-derived, inflammatory macrophages into the liver, as defined by our study, makes it an interesting drug for the treatment of NASH fibrosis but also a potential complementary partner for novel combination therapies.

Acknowledgment: We thank Sybille Sauer-Lehnen and Carmen Gabrielle-Tag for their excellent technical assistance.

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